

FINAL REPORT

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**Development of the Circadian Timing System in Rat Pups Exposed to
Microgravity During Gestation**

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PROJECT DESCRIPTION

Ten pregnant Sprague Dawley rat dams were exposed to spaceflight aboard the Space Shuttle (STS-70) for gestational days 11-20 (G 11-20; FLT group). Control dams were maintained in either a flight-like (FDS group) or vivarium cage environment (VIV group) on earth. All dams had *ad lib* access to food and water and were exposed to a light-dark cycle consisting of 12 hours of light (~ 30 lux) followed by 12 hours of darkness. The dams were closely monitored from G 22 until parturition. All pups were cross-fostered at birth; each foster dam had a litter of 10 pups. Pups remained with their foster dam until post natal day 21 (PN 21). Pup body mass was measured twice weekly. At PN14 FLT pups had a smaller body mass than did the VIV pups ($p < 0.01$). Circadian rhythms of body temperature and activity of pups from two FLT dams ($n = 8$), two FDS dams ($n = 9$) and two VIV dams ($n = 7$) were studied starting from age PN 21. All pups had circadian rhythms of temperature and activity at this age. There were no significant differences in rhythms between groups that could be attributed to microgravity exposure. We also examined the development of neural structures involved in circadian rhythmicity: the retina, the intergeniculate leaflet (IGL) and the circadian pacemaker, the suprachiasmatic nucleus (SCN). There were small differences between the flight and control groups at very early stages of development

(G 20 and PN3) which indicated that the development of both the SCN and the IGL.

These results indicate that exposure to the microgravity environment of spaceflight during this embryonic development period does not affect the development of the circadian rhythms of body temperature and activity, but may affect the early development of the neural structures involved in circadian timing.

BACKGROUND & SIGNIFICANCE

The circadian timing system (CTS) provides an organism with temporal order (for review, Moore-Ede et al, 1982). An organism's endogenous circadian rhythms are generated by neural pacemakers that both control and coordinate its physiology and behavior. These pacemakers ensure that physiological and behavioral events occur in a synchronized manner, affording the animal better adaptation to the cyclic earth environment. Proper CTS function is essential for an organism's physical and mental health (Czeisler et al, 1981; Lewy & Sack, 1986).

Circadian rhythms can be synchronized by a light/dark cycle. For a nocturnal rodent, this would result in body temperature being high during the dark (active phase) and low during the light (rest phase). Light is the critical environmental time cue or 'Zeitgeber' that allows the circadian rhythms to maintain a stable phase relationship with the 24-hour day. This process of synchronization of circadian rhythms by light is called entrainment.

The suprachiasmatic nucleus (SCN) within the hypothalamus is the neural pacemaker that generates circadian rhythms. The SCN must receive photic information in order to entrain the circadian rhythms to the light/dark cycle (Miller et al, 1996; Moore-Ede et al, 1982). There is a direct visual pathway from the retina (W cells in the cat) to

the SCN, this is the retinohypothalamic tract (RHT) (Miller et al, 1996; Moore-Ede et al, 1982; Murakami et al, 1989). In addition, there is an indirect visual pathway from the retina to the intergeniculate leaflet (IGL) of the thalamus, which relays photic information to the SCN, the geniculo-hypothalamic tract (GHT) (Miller et al, 1996).

Several studies have demonstrated that phase shifting light pulses induce c-Fos activity within SCN and intergenicualte leaflet neurons (Miller et al, 1996). Therefore, c-Fos induction within SCN and IGL neurons has become a useful marker for RHT and GHT function respectively. Our previous studies also showed that the development of c-Fos expression in SCN neurons is correlated with the formation of the outer plexiform layer of the retina and maturation of the RHT terminals in the SCN (Murakam et al, 1995).

Exposure to altered gravitational environments, either the microgravity of spaceflight or hyperdynamic fields produced via centrifugation, affects circadian rhythms (Fuller, Hoban-Higgins et al, 1994). The response of the CTS to increased gravitational fields is tripartite: an initial response is followed by a period of adaptation and the establishment of a new steady-state. The body temperature rhythm responds to 2G with an initial decrease in mean body temperature and attenuation of the rhythm. The subsequent reestablishment of the body temperature rhythm occurs over several

weeks. Exposure to timed pulses of 2G can entrain (Demaria-Pesce et al, 1991) and phase shift (Hoban-Higgins et al, 1995) the CTS. In addition to its effects on overt rhythms, exposure to 2G depresses the normal c-Fos expression in the neural pacemaker, the suprachiasmatic nucleus, in response to a light pulse (Fuller, Murakami et al, 1994). In microgravity, the body temperature rhythm of humans and non-human primates exhibits a delayed waveform with respect to other physiological rhythms (Gundel et al, 1993; Fuller et al, 1996, Monk et al 1998).

Development is affected in hyperdynamic environments. Exposing neonatal rats to a 2G field has significant effects on the morphology of the vestibular nuclei (Johnson et al, 1976). Our previous work has shown that centrifugation has significant effects on neural structures involved in circadian timing; exposure to 2G delays the development of the retina (Murakami & Fuller, 1985) and alters the pattern of development of oxidative metabolism within the suprachiasmatic nucleus (Murakami & Fuller, 1986).

In a previous spaceflight experiment (COSMOS 1514), 10 pregnant Wistar rats were exposed to microgravity for gestational days 13-18 (G13-18). Half of this population was sacrificed upon landing; the G18 fetuses were compared with those from control groups, revealing that development had proceeded normally in space, but on a delayed schedule (Serova et al, 1984). The flight fetuses exhibited a lower level of

ossification, higher water content and lower body weight than the control fetuses, all of these conditions are indicators of delayed development. Pups born to the spaceflight dams had a lower birth weight and a higher mortality rate than the control population (Alberts et al, 1986). However, these results could either be due to a direct effect of spaceflight exposure on the pups themselves, or to indirect effects of microgravity exposure via the dam (Serova et al., 1984). Indirect effects could have included a decreased maternal care resulting from microgravity exposure (Alberts et al, 1986).

In rats, the body temperature and activity rhythms develop and mature over the first 50 days of life (Kittrell & Satinoff, 1986; Cambras & Diez-Noguera 1991). In ambient temperatures below thermoneutrality, juvenile rats express a temperature rhythm that disappears during the 3rd week of life; however, this early temperature rhythm has been attributed to circadian variation in the ability of the body to defend itself against a cold stress rather than a true body temperature rhythm (Nuesslein & Schmidt, 1990). The circadian temperature rhythm matures after the thermoregulatory mechanisms needed to produce an adult-like body temperature range are in place (Kittrell & Satinoff, 1986).

In order to determine if the development of the CTS and the rhythms it generates and controls would be affected by exposure to microgravity during gestation, ten gravid

rats were flown on the Space Shuttle (STS-70) for gestational days 11-20. After their return to earth, the offspring of these rats were studied along with those from dams that had been maintained under flight-like or vivarium conditions, on the ground at earth's gravity (1 G). In order to remove the confounding variable of the effect of spaceflight on maternal care, all pups were placed with foster dams two hours after parturition.

EXPERIMENTAL METHODOLOGIES

Subjects

Twenty-four Sprague Dawley rat pups served as the subjects of this experiment. The pups were part of the NASA NIH-R2 investigation of the effects of microgravity exposure on development. The protocols followed in these experiments were approved by the Institutional Animal Use and Care Committees of Ames Research Center and UC Davis. Ten timed-pregnant dams were flown aboard the Space Shuttle (STS-70) for gestational days 11-20 (G 11- 20). These constituted the Flight group (FLT). Two control groups were established, each consisting of 10 timed-pregnant dams. One control group was maintained in a flight-like environment at Kennedy Space Center (Flight Delayed Synchronous control group; FDS). To allow for the mimicking of any unexpected event during the shuttle flight, the schedule of the FDS group was delayed 48 hours from the FLT group. A cage control group (VIV) remained in vivarium caging throughout the study and was studied on the same schedule as the FLT group. The eight FLT pups (5 male, 3 female) assigned to this study came from two litters (FLT I and FLT J), as did the nine FDS pups (4 male, 5 female) and seven VIV pups (5 male, 2 female).

Pregnant animals were received from the vendor 9 days prior to the scheduled launch of the shuttle (G 3). At gestational day 7 a laparotomy was performed under general anesthesia on each dam to confirm the number of fetuses present; only dams with 10 or more fetal buds were chosen as subjects for the experiment.

Postflight, all dams gave birth on G 22-23. All pups were cross-fostered two hours after parturition; cross-fostering took place within 8.25 hours of the foster dams' delivery. Each foster dam had 5 natural pups and 5 foster pups. Each pup was tattooed at the time it was cross-fostered and, on postnatal days (PN) 7 & 8, equipped with a microchip (IMI, BioMedic Data Systems) to allow identification. At PN 15, foster dams and their litters were shipped from Kennedy Space Center to UC Davis.

Animal husbandry

At Kennedy Space Center, all dams were maintained in a 24 hour light dark cycle consisting of 12 hours of incandescent light (approximately 30 lux) followed by 12 hours of dim red light (<1 lux; 650 ± 10 nm). FLT and FDS dams (with or without litters) were individually housed in standard vivarium cages, except for days G 12-G 20. During this time, dams were group housed ($n = 5$) in an Animal Enclosure Module (AEM). VIV dams remained in standard vivarium cages for the entire study.

From animal receipt until birth, dams were provided with the standard NASA food bar diet (Harlan Teklad TD97071) and water *ad libitum*. Food and water consumption and body weights were recorded daily preflight and postflight until G 22. Dams were left undisturbed after G 22 and monitored until birth.

At UC Davis, each foster dam and litter was housed in a standard vivarium cage set within a light-tight, sound attenuating chamber. An LD 12:12 cycle matching that under which the animals had experienced at KSC was provided (L = 30 lux; D = darkness). The timing of the LD cycle was conserved; the animals were maintained on Eastern Standard Time. After surgery, each pup was individually housed in a standard vivarium cage. Food (Purina Rat Chow) and water were available *ad libitum*. Animal husbandry was performed weekly.

Transmitter surgery

At PN 21, pups were implanted with a biotelemetry transmitter to allow for the collection of body temperature (T_b) and activity (Act) data. Initial anesthesia was performed using isoflurane in an induction chamber. Throughout the operation, a surgical plane of anesthesia was maintained using isoflurane delivered by a gas anesthesia machine through a nose cone. Under aseptic conditions, a ventral midline

incision was made through the abdominal skin and musculature. A sterile transmitter was inserted into the abdominal cavity (Minimitter, Inc., VM-FH). The abdominal musculature was sutured using non-absorbable suture; the skin was closed with absorbable suture. Animals were monitored continuously until recovery from anesthesia.

Data Collection and Analysis

Body temperature ($^{\circ}\text{C}$) was recorded at five minute intervals and activity (counts) in 5 minute bins using an antenna set beneath each cage. Data were stored on a microcomputer for later analysis. Activity counts were made as the animal moved with respect to the antenna; activity is thus a reflection of the general level of movement rather than any specific behavior. Data collection began after the animal recovered from surgical anesthesia and continued until PN 90. Pups were weighed twice weekly (on days 1 and 4 of each week of the study).

Average daily waveforms were constructed using 72 hour segments of data at weekly intervals, i.e. for days 5-7 of each week of the study. Pups were not handled or disturbed during this time. Week 4 data thus represents PN 25-27, week 5 PN 32-34 and so on, with week 9 being PN 60-62. Multiple regression was used to determine if

experimental condition, age, weight or gender contributed to differences in mean body temperature and activity levels; the following mean levels were examined: 24 hour mean, mean during the 12 hour dark phase and mean during the 12 hour light phase. Body temperature and activity data were analyzed using an adaptation of the cosinor method (Halberg, Tong et al. 1967; Halberg, Johnson et al. 1972) in which a least-squares method is used to fit a sine wave to successive 24-hour segments of the data set. For each day, the time of the calculated peak (acrophase), the amplitude of the fitted sine function, and the mean of the data are given. These analyses were used to compare relative timing, amplitude and daily mean of the rhythms between the three groups. ANOVA and a post-hoc Tukey's test was used to compare the body weights and development of the rhythms between groups ($p \leq 0.05$).

Histology

Following space flight the following pup ages were examined: G20, PN1, PN3, PN8, PN10, PN14, PN17, PN21, PN28, and PN35. All postnatal flight and control groups (1G) of rat pups were divided into two subgroups: 1) animals which received a one hour light pulse at CT13.5, and 2) animals which received no light pulse. Rats were sacrificed, retinas and brains placed in 4% paraformaldehyde in 0.1M phosphate buffer

pH 7.4, and stored at 4°C. Brains were coronally sectioned at 50 μm , immunohistochemically stained for c-Fos (Oncogene Sci.), true blue used as the chromagen, and counterstained with neutral red. Retinas were embedded in paraffin and sectioned at 8 μm (by NeuroScience Associates), and counterstained with hemotoxylin & eosin.

RESULTS

Body Mass

Figure 1 is a graph of the average male and average female pup weights for the FLT (black line), FDS (gray line) and VIV (dashed line) groups. Pups were being removed from the population at intervals to serve as subjects in other studies; therefore, the graph represents the weights from a decreasing population. After PN 35 the data represent only the pups that were studied at UC Davis. Among pups studied at UC Davis, there was a tendency for the FLT pups to be smaller than either the FDS or VIV pups. However, the groups were not significantly different in body mass.

The pups studied in this experiment were a subset drawn from the litters of 10 FLT, 10 FDS and 10 VIV dams. On PN 14, prior to their shipment to UC Davis, there were 41 FLT pups (22 male, avg mass \pm s.e. = 34.76 ± 0.83 g; 19 female, 34.39 ± 0.78 g), 45 FDS pups (21 male, 37.05 ± 0.97 g; 24 female, 35.12 ± 0.59 g) and 39 VIV pups (23 male, 36.36 ± 0.67 g; 16 female, 37.77 ± 1.11 g) extant in the population at Kennedy Space Center. ANOVA was used to compare body mass between the three groups on this day. The FLT pups (34.59 ± 0.57 g) weighed significantly less than did the VIV pups (36.77 ± 0.62 ; $F = 4.545$, $P \leq 0.013$), but did not differ in weight from the

FDS pups ($36.02 \pm 0.56\text{g}$). These results are shown in Figure 2 as a histogram of average body mass for each group.

Circadian Rhythms

Circadian rhythms in both body temperature and activity were apparent after recovery from surgery on PN 21. Raw data for temperature (upper panels) and activity (lower panels) from representative FLT (left), FDS (middle) and VIV (right) animals can be seen in Figure 3. The data plotted are from PN 25-27. Both activity and temperature are higher during the dark and lower in the light, as is normal in this nocturnal species.

Three day segments of data were analyzed at weekly intervals. This method allowed us to look at average variables and avoid days when pups were handled for weighing or cage changing. These data were analyzed for: daily mean, circadian amplitude and phase using cosinor analysis.

Body Temperature

The average 24 hour waveforms from PN 25-27 (Week 4; upper panel) and PN 46-48 (Week 7; lower panel) for a FLT (black line), FDS (gray line) and VIV (dashed line) pup are presented in Figure 4. The horizontal lines represent the mean cycle

temperature. Comparing the two panels, the maturation of the rhythm is evident in increase in the amplitude and mean and delay in the phase of the body temperature rhythm from the 4th to the 7th weeks.

The mean, amplitude and phase for the body temperature rhythm are summarized in Figure 5. There was no significant difference between the groups in mean or amplitude. However, the phase of the body temperature rhythm was significantly earlier in the VIV (20.0 ± 0.21 h) compared to the FLT (20.9 ± 0.15 h) and FDS (21.3 ± 0.14 h) groups ($p < 0.001$; $F = 22.811$). An examination of the average data for each litter revealed that this difference was due to data from just one of the VIV litters; VIV I (19.5 ± 0.28 h) was significantly different from FLT J (21.1 ± 0.37 h), FDS I (21.1 ± 0.45 h) and FDS J (21.4 ± 0.31 h; $p < 0.07$; $F = 3.990$).

The emergence of the mature temperature rhythm is indicated by the significant differences between weeks. Mean temperature for week 4 (37.5 ± 0.04 °C) was significantly different from weeks 6 (37.8 ± 0.03 °C), 7 (37.8 ± 0.04 °C), 8 (37.9 ± 0.03 °C) and 9 (37.9 ± 0.03 °C) while mean temperature for week 5 (37.6 ± 0.05 °C) differed significantly from that of weeks 7, 8 and 9 ($p < 0.001$; $F = 12.503$). Regression analysis showed that age, weight and gender all are correlated with the differences in the 24 hour mean in body temperature. These same factors are correlated with the mean

temperature over the 12 hour dark phase and, except for gender, the mean temperature achieved during the 12 hour light phase. The amplitude of the temperature rhythm differed significantly between week 4 (0.3 ± 0.02 °C) and weeks 7 (0.5 ± 0.02 °C), 8 (0.5 ± 0.03 °C) and 9 (0.5 ± 0.03 °C), between week 5 (0.3 ± 0.02 °C) and weeks 6 (0.4 ± 0.02 °C), 7, 8 and 9 and between weeks 6 and 9 ($p < 0.001$; $F = 20.181$). The phase of the temperature rhythm was significantly earlier in week 4 (19.4 ± 0.16) than all subsequent weeks (5 = 20.5 ± 0.26 h; 6 = 21.1 ± 0.20 h; 7 = 21.1 ± 0.23 h; 8 = 21.3 ± 0.19 h; 9 = 21.3 ± 0.21 h; $p < 0.001$; $F = 17.376$).

Activity

The average daily waveforms of the activity rhythm for the time interval PN 25-27 (upper panel) and PN 46-48 (lower panel) shown in Figure 6. Data are plotted from a representative FLT (black line), FDS (gray line) and VIV pup (dashed line). The horizontal lines give the respective daily mean activity levels for each pup. In contrast to the body temperature rhythm, mean activity levels decreased over time and the amplitude of this rhythm did not change over the course of the study.

The mean, amplitude and phase of the activity rhythm are summarized in Figure 7. In contrast to the temperature rhythm, the mean level of the activity rhythm did show a significant difference between groups, with the FDS having a higher mean ($30.5 \pm$

0.58) than the FLT (26.6 ± 0.72 ; $p < 0.001$; $F = 10.408$). The activity rhythm of the FDS group had a higher amplitude (21.0 ± 0.69) than either FLT (16.2 ± 0.65) or VIV (17.8 ± 0.58 ; $p < 0.001$; $F = 15.476$). The phase of the activity rhythm for FDS (21.4 ± 0.8 h) was significantly later than that of the VIV group (21.14 ± 0.11 h, $p < 0.039$; $F = 3.328$).

Examining the development of the rhythm over time, mean activity levels for weeks 4 (30.2 ± 1.15) and 5 (30.8 ± 1.02) were significantly greater than those from week 9 (25.7 ± 0.79 ; $p < 0.002$; $F = 4.025$). Mean activity levels over both the 24 hour day and the 12 hour dark phase were correlated with both group and age; mean activity levels during the 12 hour light phase were correlated with weight. The amplitude of the activity rhythm did not change over time; there was no significant effect of week of study on this variable. There was a significant difference in the phase of the activity rhythm between week 4 (21.9 ± 0.15 h) and weeks 5 (21.1 ± 0.12 h), 6 (21.1 ± 0.13 h), 7 (21.3 ± 0.08 h) and 9 (21.1 ± 0.15 ; $p < 0.001$; $F = 6.798$).

Neural Development

Retina

The development of the retinal laminae from rat pups was compared between flight and 1G control groups. The retina (Figure 8) of a mature rat exhibits a number of

distinct laminae that have a stereotypical pattern of development. For example, the appearance of the outer plexiform layer normally occurs by PN5 and coincides with the development of RHT function. At the early ages examined (G20, PN1, PN3, and PN8), there were no differences in the measurement of laminar thickness or the timing in the appearance of any specific layer between the flight and control groups.

Suprachiasmatic Nucleus

During development many neurons throughout the brain exhibit a transient increase in c-Fos activity in a stereotypic pattern that is specific for each neural structure. The G20 Controls exhibited c-Fos activity in a small group of SCN neurons confined along the medial border. This pattern of restricted c-Fos activity in the SCN is not present at PN1. However, the flight group at G20 exhibits c-Fos positive neurons that are more widely distributed across the SCN. Instead of being confined to the medial portion of the SCN, a large number of c-Fos positive neurons were found in the lateral and dorsal regions. However, this dispersed pattern of c-Fos activity in the SCN of the G20 flight group is not present at PN1. This suggests that the SCN of the G20 flight group is significantly delayed in development, but no differences were found by PN1.

Adult rats exhibited a significant induction of c-Fos activity within SCN neurons in response to a one hour phase shifting light pulse (Figure 9). Rats not exposed to a light pulse during the dark period exhibited few c-Fos reactive neurons within the SCN (Figure 9A). However, following a one hour light pulse during the early dark period, there were prominent c-Fos reactive neurons located within the ventral portion of the SCN (Figure 9B). The development of c-Fos induction within SCN neurons in response to a phase shifting light pulse was compared between control and flight groups. For both the control and flight groups, a light pulse did not induce any c-Fos activity within the SCN at PN1 or PN3. However, at PN8 there was a robust induction of c-Fos by the light pulse for both control and flight groups.

Intergeniculate Leaflet

Adult rats exhibited significant c-Fos induction within IGL neurons following a phase shifting light pulse. The development of c-Fos induction within IGL neurons in response to a phase shifting light pulse showed significant differences between the control and flight groups. A one hour phase shifting light pulse did not induce any c-Fos activity within the IGL at PN1 for both control and flight groups. For the controls the earliest age when phase shifting light pulse could induce c-Fos activity within the IGL neurons occurred at PN3. At PN8 there was a robust induction of c-Fos within the IGL

neurons by the phase shifting light pulse for the control group. By contrast, the one hour phase shifting light pulse did not induce c-Fos activity within the IGL neurons in any animal from the PN3 flight group. However, by PN8 there was a robust induction of c-Fos in the IGL neurons following the phase shifting light pulse for flight group. This would suggest that at PN3 the flight group exhibited a delay in the development of this important circadian visual pathway. However, by PN8 the flight group had recovered from the delay in development.

CONCLUSIONS

In a previous spaceflight experiment (COSMOS 1514), rats were exposed to microgravity during gestational days 13-18 (Mains & Gomersall, 1986). The flight dams, pups and fetuses were examined and compared with control rats that had remained on earth. On landing, the spaceflight dams had a lower body mass than did the controls. In addition, Serova et al (1984) determined that fetuses from this experiment showed delays in development, including an increased water content, lower ossification level and smaller body weight. The experimental pups were more variable in body weight and had a higher mortality than control pups that had not been exposed to microgravity during gestation (Alberts et al., 1986). However, it could not be determined if this was due to spaceflight effects on the pups or to a decreased maternal care by the dams. These pups also exhibited a delay in the development of the response to 40 kHz tones, and there was a suggestion that they were more sensitive to rotation.

In our study, the pups exposed to the microgravity environment of spaceflight from G11-20 had a lower body weight than either group of control pups. On PN 14 the FLT pups weighed significantly less than did the VIV control pups. In the subset of pups that were studied at UC Davis, the difference in mass between the experimental and control groups was maintained through 90 days of age, although not at a significant

level. Wong and DeSantis (1997) studying pups that had been exposed to microgravity from G9 to G20 (NIH-R1) had similar findings. Their flight animals had a lower average daily weight gain than did controls. However, the flight population studied by Wong and DeSantis (1997) had a larger ratio of female to male pups than did the control population. Sexual dimorphism was enough to account for the observed differences in weight gain. The difference in body size in both this current and the Wong and DeSantis studies could not be due to decreased post-natal maternal care resulting from spaceflight exposure, as all pups had been cross-fostered at birth. In the current study, as also had been seen previously (Serova et al., 1984), on their return to earth the FLT dams weighed significantly less than did the control FDS dams. A decrease in muscle and bone mass and a loss of body water are normal physiological responses to a decreased gravitational load (Nicogossian et al, 1993). In addition, the FLT dams had a significantly greater mg of adrenal mass per g of body mass than did the FDS dams (0.233 ± 0.013 mg/g vs. 0.189 ± 0.014 ; t-test; $p < 0.05$), which could have affected birth weight of the pups (NASA NIH-R2 Post Flight Report).

Although the phase of the body temperature rhythm was significantly earlier in the VIV group than in the FLT and FDS groups, this difference remained steady as the rhythms developed over the course of this study, indicating that there was no difference

in the development of an adult timing of the temperature rhythm between the three groups. An animal whose pacemaker has a shorter endogenous period may have an earlier phase than one with a longer period; the period of the pacemaker is genetically determined (Knopka, 1971; Ralph & Menaker, 1988). Both the FLT and the FDS pups were the offspring of only 2 dams, therefore a genetic component would have a larger impact than if each pup had a different mother.

The FDS group had a higher mean activity level than the FLT group and a higher activity amplitude than either the FLT or VIV groups. Pups from one of the FDS dams consistently had the highest mean activity each week of the study and, except for week 4, the highest activity amplitude. These observations again suggest the possibility that a genetic component is involved in the higher activity levels of this particular litter. No litter consistently displayed the lowest mean activity or the lowest activity amplitude.

The flight group of rats exhibited a more dispersed pattern of c-Fos activity within the SCN at G20 and failed to induce c-Fos activity within IGL neurons by a phase shifting light pulse at PN3 relative to the controls. The dispersed pattern of c-Fos neurons within the SCN suggests that the circadian pacemaker of the flight group is developmentally delayed. In addition, since a light pulse at PN3 did not induce c-Fos within the IGL, the function of the GHT also appears to be developmentally delayed.

There were no differences between flight and control groups in the development of RHT function using c-Fos as the biological marker found in this study. However, this could be due to the restricted sampling at several critical developmental periods. We have previously demonstrated that a phase shifting light pulse is able to induce c-Fos activity within the SCN at PN5 (Murakami et al, 1995), an age which could not be examined in this study. Therefore, it is possible that differences in the SCN between the control and flight groups could have been found at PN5.

There were also no differences between Flight and Control groups in the development of retinal lamination. We have previously demonstrated that the appearance of the outer plexiform layer occurs at PN5 (Murakami et al, 1995), which was not examined in this study. Therefore, it is possible that there is a delay in the development of retinal lamination in the flight group in which the outer plexiform layer would not have formed by PN5.

Both control and flight animals exhibited the same level of neural maturation within the CTS by PN8. This would suggest that prenatal exposure to microgravity results in a temporary delay in the development of the CTS, but recovery from this deficit will be achieved by PN8 if they are postnatally reared at 1G. Developmental deficits may have been more severe if exposure to microgravity was extended into

postnatal periods. In addition, it will be important to examine ages between PN3 and PN8 to determine the exact timing of neural recovery.

The delay in the development of the CTS in the flight group may be due to several factors. The microgravity environment may play a role in normal fetal development. However, it is also feasible that the effect of microgravity on fetal development is indirect. Microgravity produces a number of physiological and behavioral (e.g. stress, calcium loss) changes in the Dam that could affect the developing fetus. It will be important to determine which physiological adaptations by the Dam to microgravity exposure causes a delay in the development of the fetus.

One aspect of the study that should be addressed is the possibility that minor differences in the development of circadian rhythms by the pups may have been obscured by the provision of a light-dark cycle and a foster dam and foster siblings. Stern and Levin (1975) have shown that the dams influence the feeding rhythms of the pups. Dams fed ad lib or only during the night had a diurnal pattern of maternal behavior and litter weight gain while dams fed exclusively during the day had nocturnal patterns (Stern & Levin, 1975). Sighted dams impose a diurnal milk intake pattern on their offspring, whether the offspring are sighted or blind (Levin & Stern, 1975).

Our conclusion is that spaceflight exposure during this period of gestation, while it does result in minor developmental delays, does not markedly affect mammalian development. In this study, rat pups exposed to microgravity from G11-G20 developed normal, mature circadian rhythms that were comparable to those of control rat pups. These observations agree with those of previous spaceflight experiments in which rat pups were exposed to microgravity during gestation (Serova et al, 1984, Alberts et al, 1986, Wong & DeSantis, 1997).

APPENDIX A: FIGURES

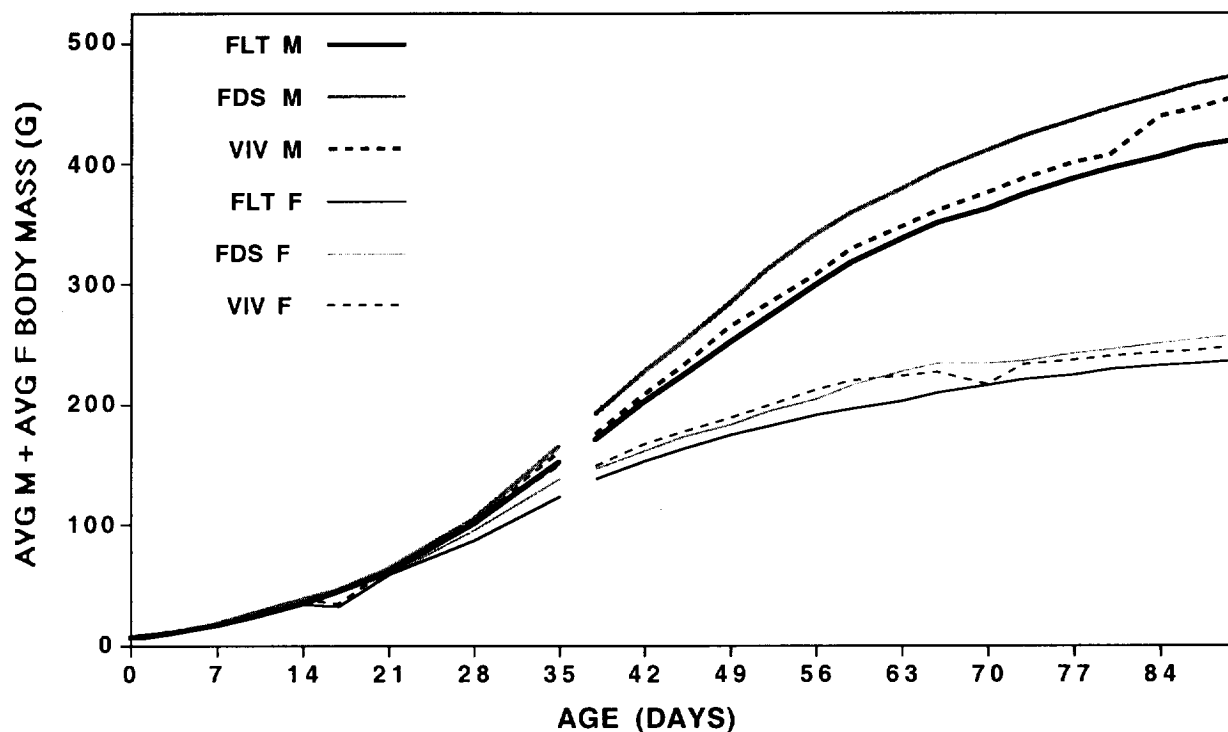


Figure 1: The average body mass for males and females is plotted vs. age for the FLT (black line), FDS (gray line) and VIV (dashed line) populations. At the start of the study, there were 67 FLT pups (35 male, 32 female), 70 FDS pups (35 male, 35 female) and 62 VIV pups (36 male, 26 female). From PN 1 to PN 35, pups were culled from each group to serve as subjects in other studies; after PN 35, the body mass data comes solely from the subset of pups studied at UC Davis.

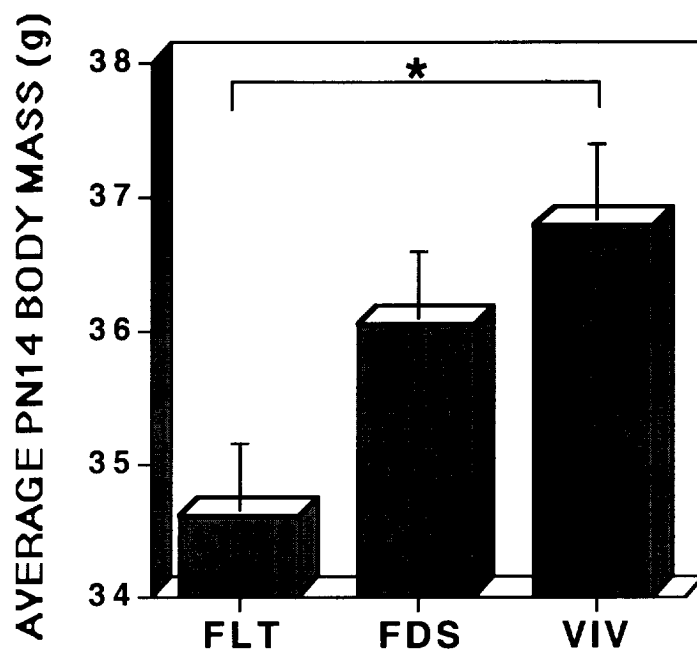


Figure 2: Histogram of average body mass (+s.e.) of FLT, FDS and VIV pups on PN14. (* $P \leq 0.01$).

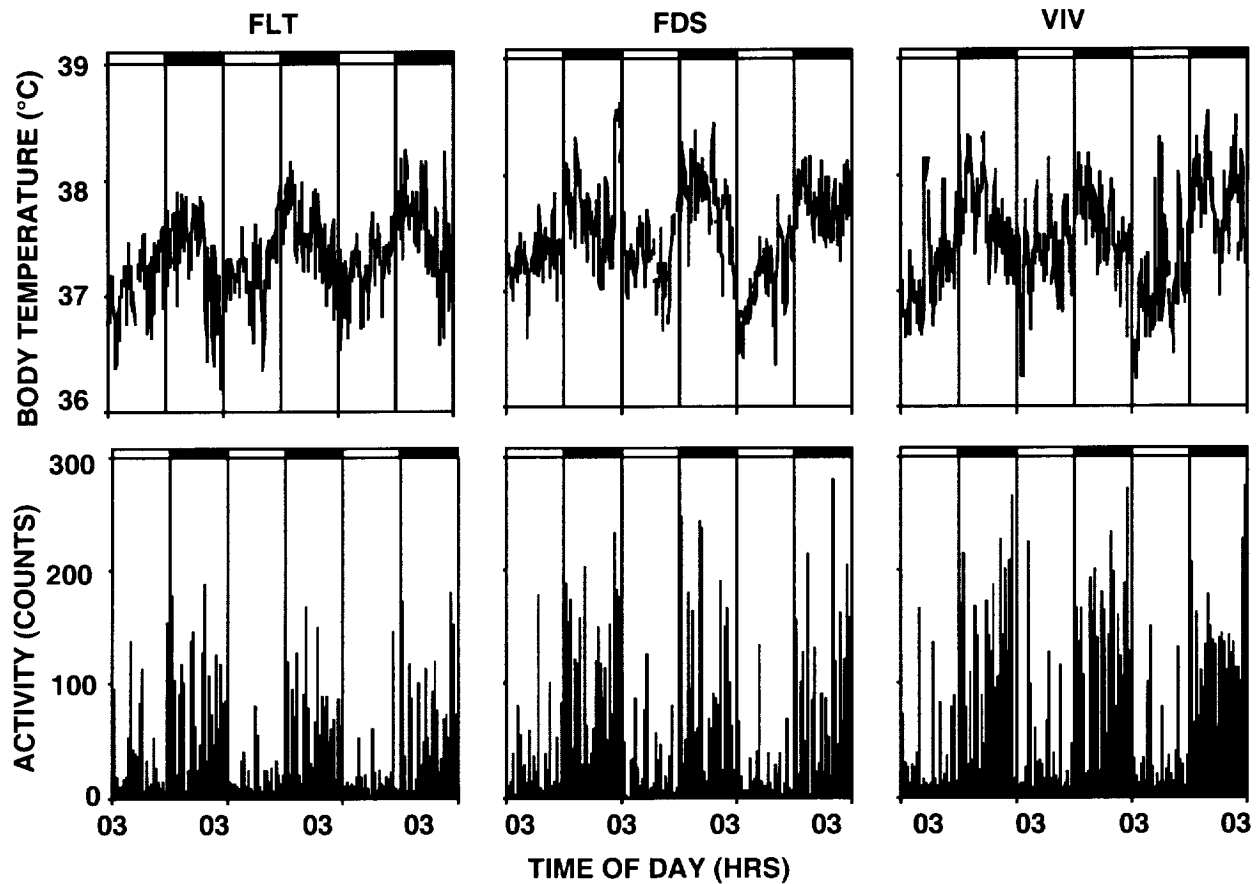


Figure 3: Raw data from a representative FLT (left), FDS (middle) and VIV (right) pup. Body temperature ($^{\circ}\text{C}$; upper panels) and activity (counts/5 minutes; lower panels) are plotted vs. time of day for days of age PN 25-27. The light-dark cycle is represented by the bars of light and dark at the top of the graphs. All animals show circadian rhythms in both temperature and activity, with higher values occurring during the dark portion of the light-dark cycle, as is normal in this nocturnal species.

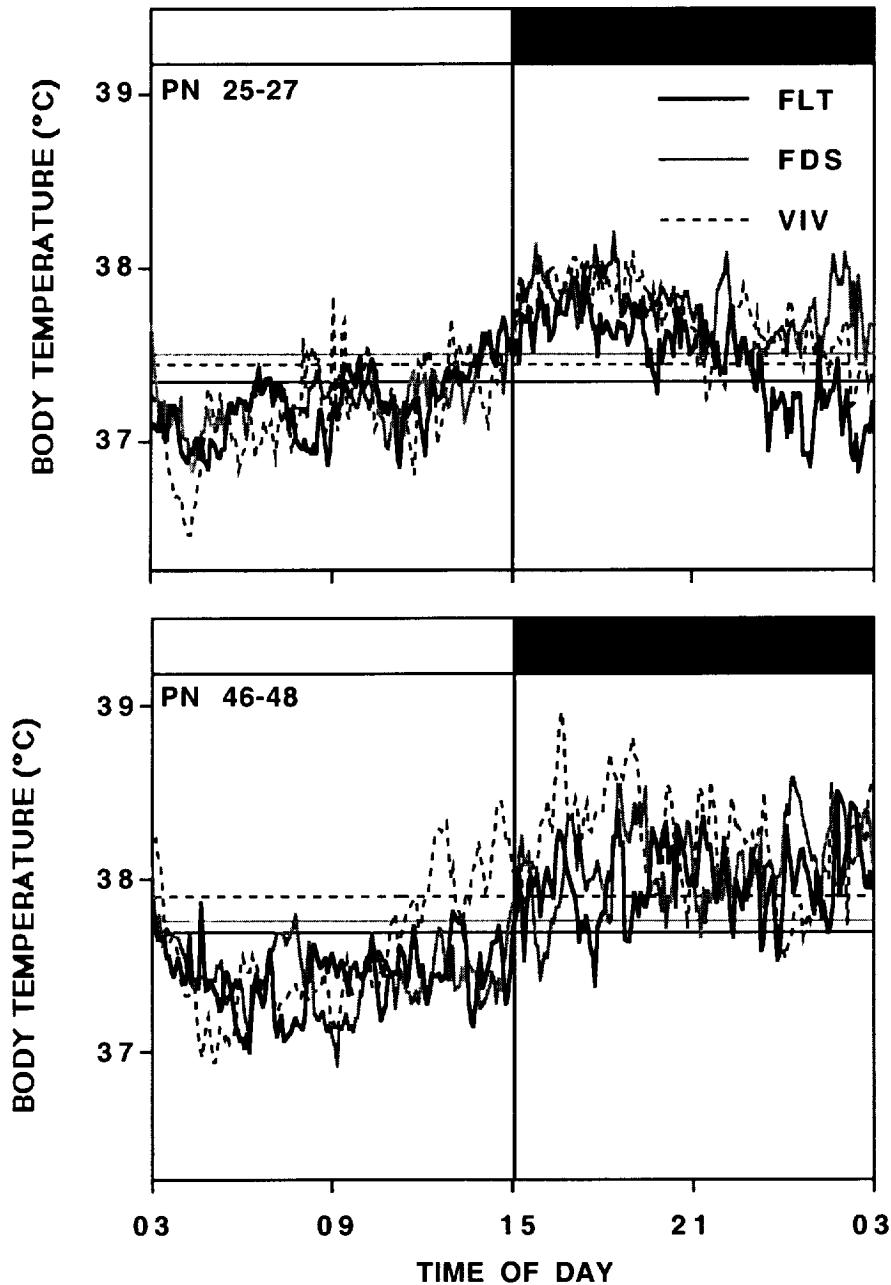


Figure 4: The average 24 hour waveforms of body temperature for a FLT (black line), FDS (gray line), and VIV (dashed line) animal for days PN25-27 (week 4; upper panel) and PN46-48 (week 7; lower panel). The light-dark cycle is represented by the light and dark bars at the top of the graphs. The mean body temperature levels are represented by the horizontal lines.

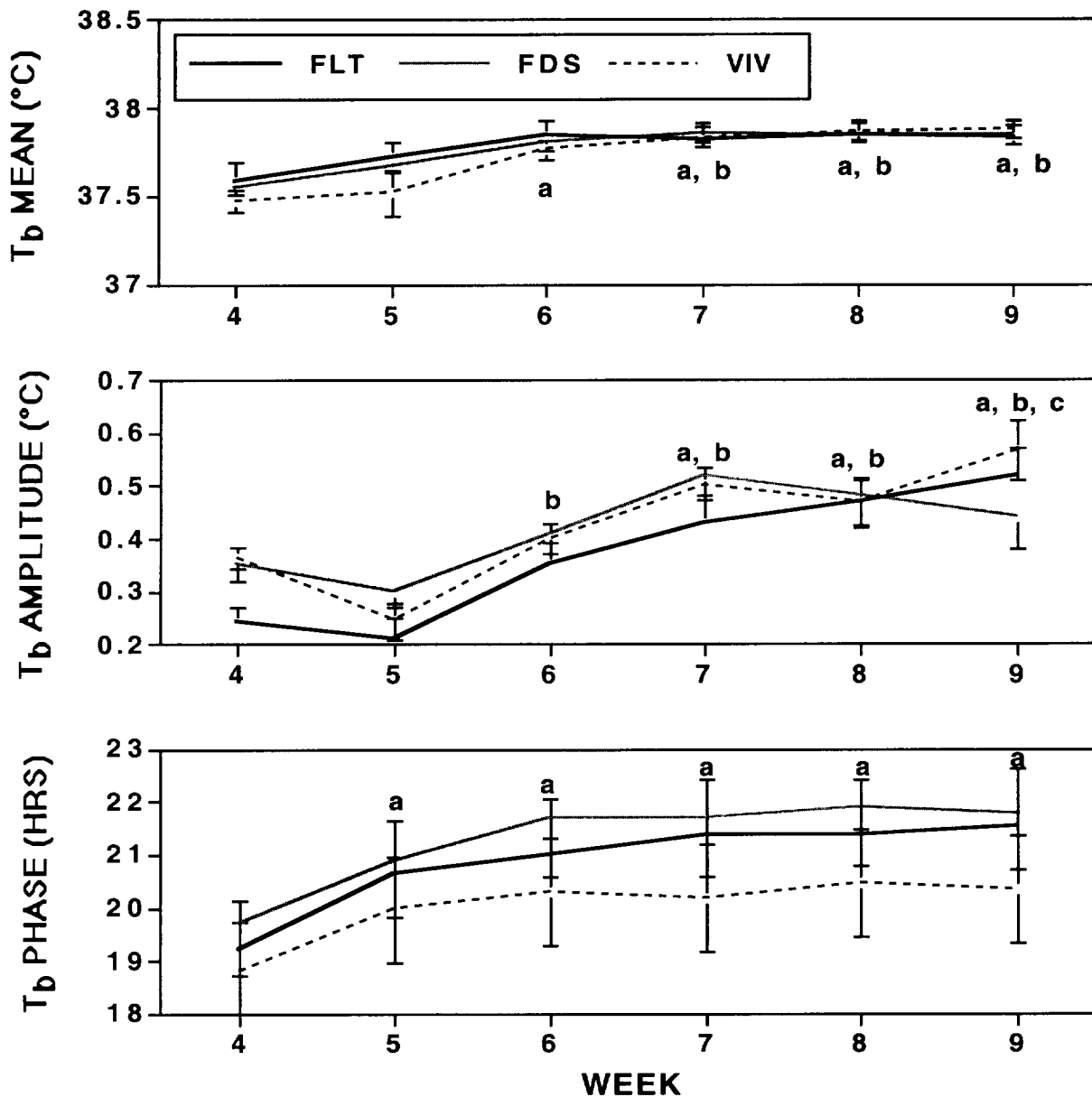


Figure 5: Body temperature mean ($^{\circ}\text{C}$; top), amplitude ($^{\circ}\text{C}$; middle) and phase (time of day; lower) derived from cosinor analysis are plotted vs. week of the study for the FLT (black), FDS (gray) and VIV (dashed line) pups. These data are averages from the last 3 days of each represented week. For data within each panel:

- (a) indicates that data for this week is significantly different from that of week 4
- (b) indicates that this data point differs significantly from week 5
- (c) is used to indicate a significant difference from week 6.

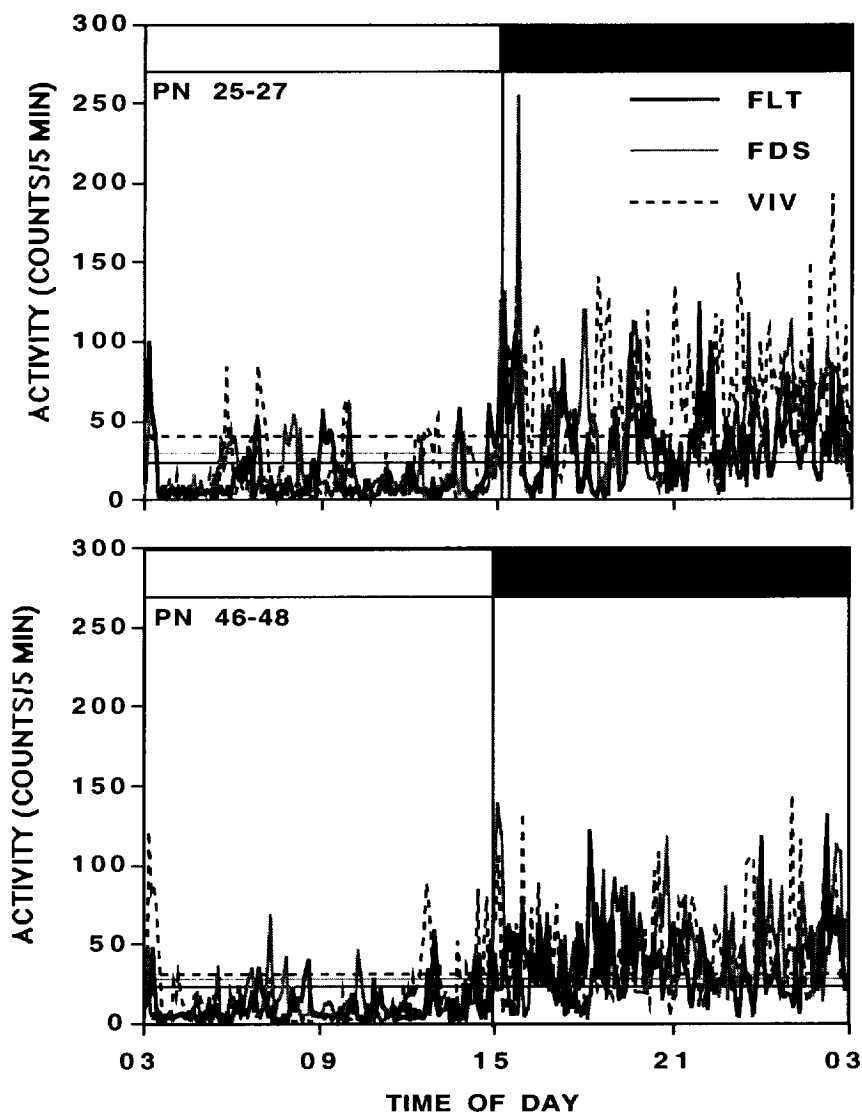


Figure 6: Activity data are presented in the same format and for the same time frame as described for Figure 4.

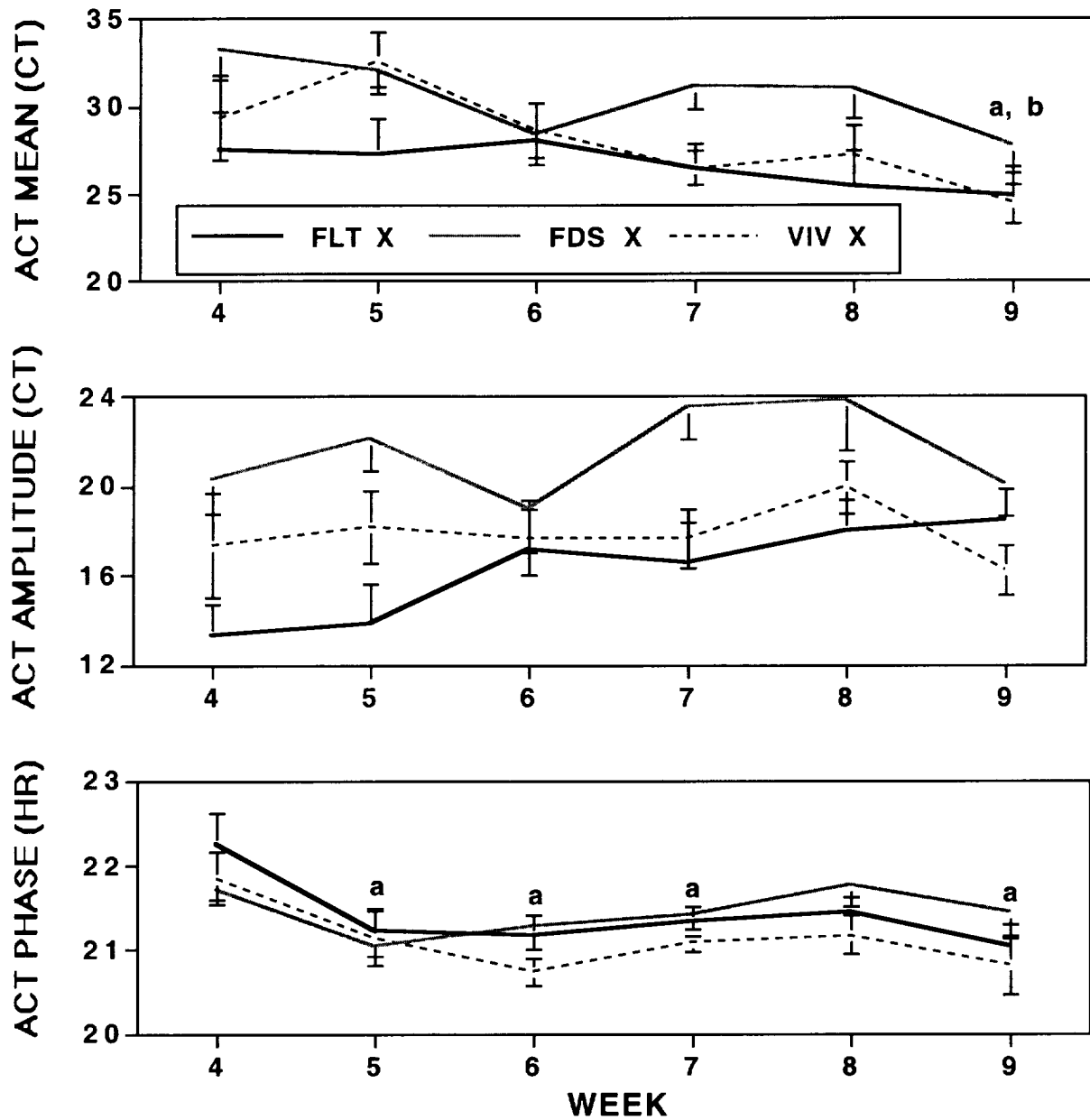


Figure 7: Activity rhythm mean (counts/5 minutes), amplitude (counts/5 minutes) and phase (time of day) are plotted vs week of the study for FLT (black), FDS (gray) VIV (dashed line) pups. Details are as in Figure 5.

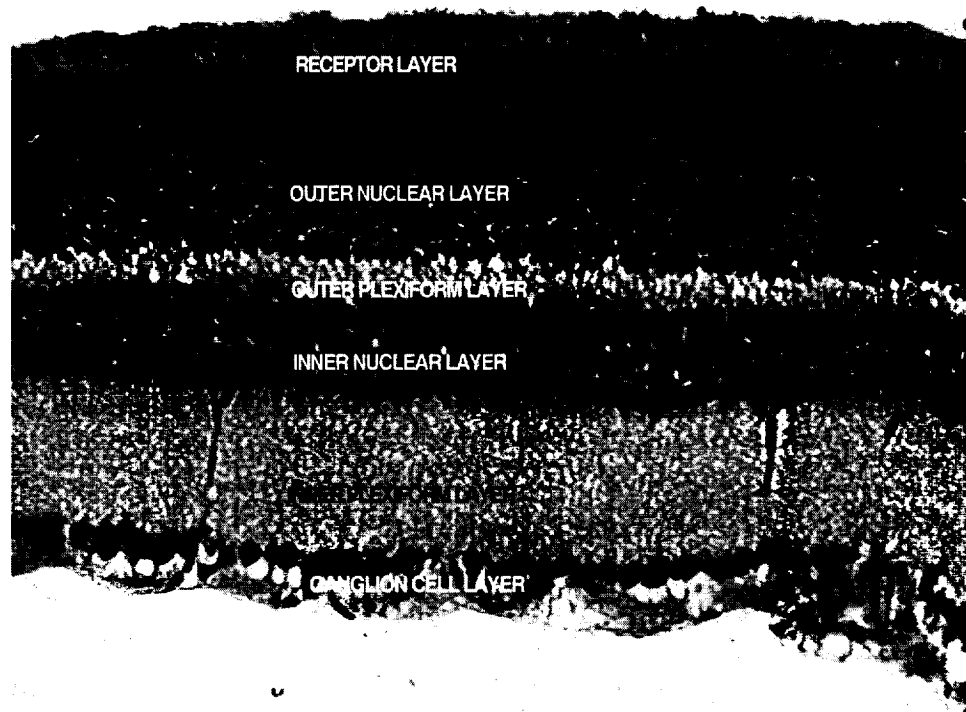


Figure 8. A cross-section of the retina of P28 rat demonstrating the distinct borders between laminae.

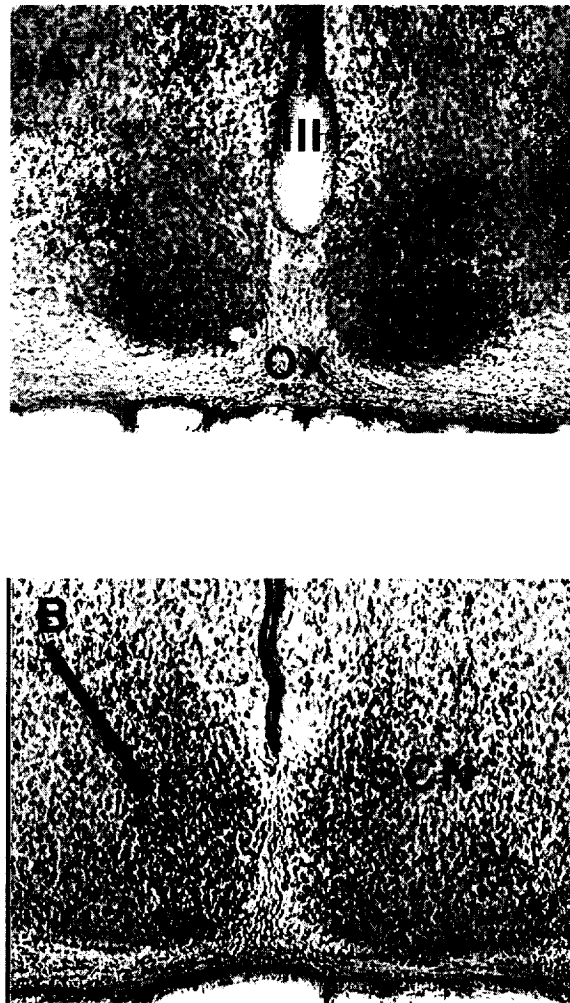


Figure 9. **A.** Section through the SCN of a rat that was not exposed to light. There are very few neurons within the SCN that exhibit c-Fos reactivity. **B.** Section through the SCN of a rat that has been exposed to a 1 hour light pulse. There are significant numbers of c-Fos reactive neurons (arrow) predominately located in the ventral portion of the SCN (III, third ventricle; OX, optic chiasm).

APPENDIX B: LITERATURE CITED

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